Mouse Ascites Fluid as a Source of Antibody Against Molluscan Antigens

BY

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(4 Plates; 4 Text Figures; 21 Tables)

INTRODUCTION

Utilization of immunological techniques as applied to molluscan systematics has recently increased. This trend is seen in studies on Lymnaeidae (Tran Van Ky *et al.*, 1962; Morrill *et al.*, 1964), Planorbidae (Tran Van Ky *et al.*, 1962; Michelson, 1966a,b; Wright & Klein, 1967; Burch, 1968), Hydrobiidae (Davis, 1968a), Pleuroceridae (Davis, 1968b, 1969), and Pelecypoda (Fisher, 1969). These studies have involved precipitating antigen-antibody systems in ring-tests, Ouchterlony diffusion plates, acrylamide and agar gel immunoelectrophoresis.

A problem exists in producing quantities of antisera for studies involving small gastropods. With whole-body extracts excluded for the reasons stated by Davis & Lindsay (1967) it is difficult to procure adequate protein to induce antibody production at a desired level using snails of the size of most Hydrobiidae or smaller. From 28 to 212 mg protein have been used to hyperimmunize one rabbit (Davis, 1969) with maximum yields of 100 ml of antiserum (pooled from two successive bleedings). With *Oncomelania hupensis nosophora* foot muscle extract, this amount of protein may involve 400 to 2000 snails respectively. It may take four rabbits to obtain one which will produce excellent antiserum; i.e., a total investment of 1600 to 8000 snails.

Munoz (1957) reported production of specific antibody of high titer in large amounts of peritoneal fluid by injecting mice with albumin antigens mixed with Freund's adjuvant. Herrmann & Engle (1958) coupled viral immunization with Sarcoma 180 cell-induced ascites to produce large volumes of peritoneal fluid with high titer antibody. Kasel *et al.* (1959) utilized a bacterial-adjuvant mixture to provide a high titer viral antibody. Lieberman *et al.* (1961) showed that mice strains varied in potential to produce ascitic fluid when inoculated intraperitoneally with *Staphylococcus aureus*—incomplete Freund's adjuvant mixtures. Sartorelli *et al.* (1966) used Sarcoma 180/TG in addition to human serum albumin, globulin and viral antigens to prepare hyperimmune ascitic fluid in mice.

The purpose of this paper is to present data showing that high titer antibody may be routinely produced in large volumes of mouse ascitic fluid when mice are injected intraperitoneally with snail antigens. Methodology is presented on how to obtain maximum yields of ascitic fluid using Sarcoma 180 cells coupled with Freund's complete adjuvant. Methods for determining the quality of the ascites are given. Antibodies (quality and quantity) in mouse ascitic fluid are compared with those in hyperimmune rabbit serum by comparing the antigen-antibody precipitating systems of 10 snail taxa with the homologous anti-*Semisulcospira libertina* systems. Specificity of antibodies in rabbit serum and ascitic fluid is analyzed and discussed. The pertinence of these findings relative to molluscan systematics and analyses of molluscan genetic systems is discussed.

MATERIALS and METHODS

1. Source of Snails:

Eleven species of prosobranch snail were utilized in this study. These are listed systematically in Table 1.

Table 1

Systematic arrangement of snails utilized in this study together with the collection localities and date of lyophilization of foot-muscle extracts.

	Systematic arrangement	Locality where collected	Date of lyophilization
	ORDER ARCHAEOGASTROPODA		
S. Family	Neritacea		
Family	Neritidae		
1)	*Clithon retropictus* (v. Martens)	Japan, Honshu, Shizuoka Pref., Shimoda	19 December 1968
	ORDER MESOGASTROPODA		
	Archaeotaenioglossa		
	Viviparidae		
2)	*Sinotaia histrica* (Gould)	Japan, Honshu, Saitama Pref., Yagyu	12 September 1968
	Rissoacea		
	Hydrobiidae		
3)	*Oncomelania hupensis nosophora* (Robson)	Japan, Honshu, Yamanashi Pref., Kofu Valley	25 April 1967
	Cerithiacea		
	Potamididae		
4)	*Batillaria multiformis* (Lischke)	Japan, Honshu, Kanagawa Pref., Manazuru	12 July 1969
	Cerithiidae		
5)	*Clypeomorus humilis* (Dunker)	Japan, Honshu, Kanagawa Pref., Manazuru	12 July 1969
	Pleuroceridae		
6)	*Semisulcospira libertina* (Gould)	Japan, Honshu, Shizuoka Pref., Shimoda	6, 9 June 1969 6, 15 August 1969
7)	*Semisulcospira niponica* (Smith)	Japan, Honshu, Shiga Pref., Lake Biwa	2 June 1969
	Thiaridae		
8)	*Brotia costula episcopalis* (Lea)	Malaysia, W. Malaysia, Selangor, Ulu Langat	31 March 1969
9)	*Stenomelania crenulata* (Deshayes)	India, Madras State, S. Arcot Dist., Coleron River	
10)	*Melanoides tuberculatus* (Müller)	Japan, Okinawa, Motobu-cho, East of Higashi	11 April 1968
11)	*Thiara scabra* (Müller)	Japan, Okinawa, Onna-Son, West of Atsuta	15 April 1968

2. Mice and Rabbits:

The strain of mouse used was 406 Inbred Swiss Albino. The animals were 18 to 23 g virgin female and/or male. Rabbits were 6 to 7 lb. virgin females of an albino strain (406 Inbred).

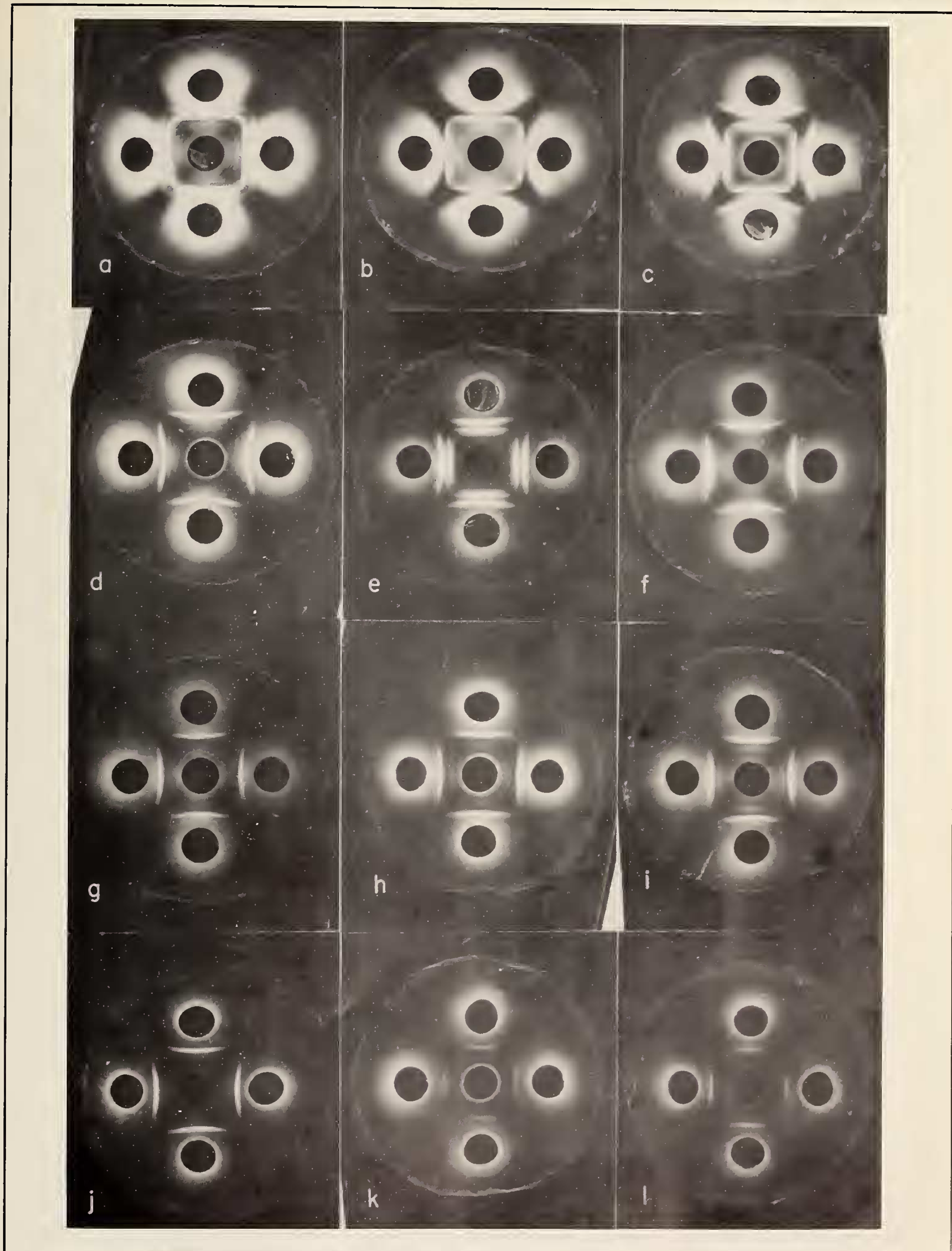
3. Preparation of Extracts:

Davis & Lindsay (1964) initiated the use of foot-muscle extracts for biochemical studies of Mollusca. Such extracts have subsequently been used by Davis (1967), Davis & Lindsay (1967), Davis (1968a,b, 1969), and Burch (1968) in electrophoretic or immunological studies, or both. Freshly cut foot muscle, trimmed of epidermis as well as pockets of dense green pigment (where these existed) was homogenized (300 mg blotted wet weight per 2.0 ml Carriker's 1946 saline) first by using a motor-driven tissue grinder with a teflon-tipped pestle. The homogenate was then transferred to a 5.0 ml micro cup and homogenized for 60 seconds at 50,000 rpm (Sorvall microhomogenizer).

Plate Explanation

Figure *1*.

Determination of the quality of immune ascitic fluid using micro-Ouchterlony double diffusion methods. a-e are "strong"; f-i are "medium," while j-n are "weak." The strength of reactions in a, b is much greater than reactions seen to date with rabbit sera with titers of 1:1024. Such "strong" ascitic fluid can be diluted 1:2 with saline (0.45%) to obtain optimal results in experiments.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All operations were carried out at 2–5°C maintained by use of ice baths. The final homogenate was centrifuged at 3000 rpm (1500 × g) for five minutes and the supernatant was decanted; the sediment was centrifuged again at 4000 rpm (2600 × g) for five minutes. The supernatants were combined.

Protein contents of pooled extracts were initially determined using the Biuret reaction (*fide* KABAT & MAYER, 1961). The standard curve was made using crystalline bovine albumin (clinical pathology standard). Subsequently, the Folin-reagent test was used (DAUGHADAY *et al.*, 1952) and a standard protein solution of crystalline bovine albumin was used with each test. The latter procedure proved more desirable as only 0.1 ml extract was needed contrasted with 1.0 ml for the former test. A Bausch and Lomb Spectronic 20 spectro-photometer was used.

Extracts were used immediately, or lyophilized in 1.0 ml units and stored until used (–20°C). Lyophilized extracts were reconstituted with distilled water.

4. Immunological Procedures:

A. Production of Antiserum—Antisera were produced in rabbits against antigens in freshly prepared or lyophilized extracts from *Semisulcospira libertina*. Control sera were obtained prior to intravenous (ear route) injections of extracts following the schedule given in Table 2. Bleeding from the ear (modified method of NACE & SPRADLIN, 1962) was conducted on two consecutive days starting on the third or fourth day after the last injection of extract. After the first bleeding each rabbit was injected with 25 ml physiological saline. Bleeding on the second day was initially by ear, then by heart puncture. After clotting and centrifugation, sera from all bleedings for one rabbit were pooled. Titers were determined by interfacial ring test and a twofold dilution series of antigen where a volume of antigen equal to that of antiserum was layered over the latter in a glass capillary tube (1.85 × 72.5 mm). The tubes were maintained at 23° ± 2°C and examined for the precipitin band at 30 and 60 minutes. The antisera used were "excellent" as defined by DAVIS (1968b, 1969). Sera were, for the majority, frozen and stored at –20°C until used.

B. Production of Ascitic Fluid—Injection and ascites collection schedules for mice are presented in tabular form (Tables 3–16) for each of eight experiments conducted for the purpose of establishing the best method of producing large volumes of mouse ascitic fluid with high titer antibody. The experiments show the effects of different techniques and injection time schedules on the volume of ascites produced. Freund's complete adjuvant was used

Table 2

Schedule for intravenous injection of five rabbits with freshly prepared or lyophilized foot-muscle extract of *Semisulcospira libertina* and the quantity and titers of sera obtained.

	Fresh extract			Lyophilized extract	
Day	A	B	C	D	E
1	2 mg	2 mg	2 mg	2 mg	2 mg
2	2	2	2	2	2
3	2	2	2	2	2
4	2	2	2	2	2
5	2	2	2	2	2
6	2	2	2	2	2
7	—	—	—	2	2
Rest	3 weeks	3 weeks	3 weeks	3 weeks	3 weeks
1	2 mg	2 mg	2 mg	2 mg	2 mg
2	2	2	2	2	2
3	2	2	2	2	2
4	2	2	2	2	2
5	2	2	2	2	2
6	2	2	2	2	2
7	—	—	—	2	2
Total injected	24 mg	24 mg	24 mg	28 mg	28 mg
10[2]	b[1]	b	b	—	—
11[2]	b	b	b	b	b
12[2]	—	—	—	b	b
Volume serum obtained	90 ml	100 ml	90 ml	90 ml	80 ml
Titer:					
30 min	1/512	1/512	1/512	1/256	1/256
60 min	$\geq$1/1024	$\geq$1/1024	$\geq$1/1024	1/512	1/512

[1]Bleed

[2]Pool sera from two days bleeding

(Difco Laboratories, Detroit, Michigan) by mixing thoroughly with an equal volume of either extract or Cariker's saline. Sarcome 180 cells were used in Experiments 4 to 8 to induce ascites production. The cell line was obtained from the Department of Virology, 406 Medical Laboratory, and maintained by injecting several mice each week with 0.5 ml of a 10% suspension of cells obtained from the previous week's injected mice. In Experiments 5 through 8, 0.5 ml of a 10% suspension in phosphate buffered saline were injected intraperitoneally.

Mice were "tapped" (paracentesis of the literature) with an 18 gauge needle whenever the mice appeared swollen. The volume per mouse per tap (one t-unit) was recorded. The ascitic fluid was allowed to clot at room

temperature for 30 minutes and under refrigeration for an additional 30 to 60 minutes. Each "t-unit" was centrifuged at 4000 rpm (2600 × g) for 10 minutes. It was evident after the fifth experiment that this procedure was not satisfactory as clotting was not complete as evidenced by further clotting after several days when the ascitic fluid was unfrozen from -20°C storage. In Experiments 7 and 8 (also used, in part, in Experiment 6 as discussed below), freshly drawn rabbit blood was added to each "t-unit" (one part rabbit blood to two parts ascitic fluid). The resulting clot cleared the ascites of all clotting elements. The dilution with rabbit blood (serum) did not seem to alter the strength or number of precipitating antigen-antibody systems.

Poor results, using the interfacial ring test to determine titer, caused us to abandon this technique for ascitic fluid. Subsequently, the worth of each "t-unit" for immunological studies was determined by micro-Ouchterlony double diffusion plates (five-hole system discussed in DAVIS, 1968a, 1969). Each "t-unit" was characterized as strong, medium or weak depending on the number of precipitation systems clearly observed in the diffusion plates in the homologous reaction. In this system, "t-units" with three or more clearly discerned systems were considered strong, those with two clearly discerned systems with or without other poorly resolved systems were classed as medium while zero or one clearly discerned system with or without poorly resolved ones were classed as weak. Pools were made of the strong and medium "t-units" but the weak units were discarded. In Experiment 6, the "strong" "t-units" were pooled and then divided into two groups only one of which received whole rabbit blood (R). Immunoelectrophoretic experiments were conducted using these "R" and "No-R" "strong" ascites pools to discern if different results would be obtained.

C. Immunoelectrophoresis—The procedures used were those given in detail by DAVIS (1968b, 1969). Over 50 immunoelectrophoretic experiments were conducted using more than 600 slides. The extracts were adjusted to a protein concentration of 6 mg/ml. All taxa of snails were compared with the homologous precipitin systems of *Semisulcospira libertina*. Immunoelectrophoretic experiments, where the homologous reactions were not as clear as the established patterns for a given serum, were discarded and those experiments were repeated. In this series of comparisons no studies with absorbed sera were conducted. Results were recorded in terms of the number of precipitin systems in the homologous reactions, number of systems in the heterologous reactions and number of systems "unique" to the homologous systems. In addi-

Plate Explanation

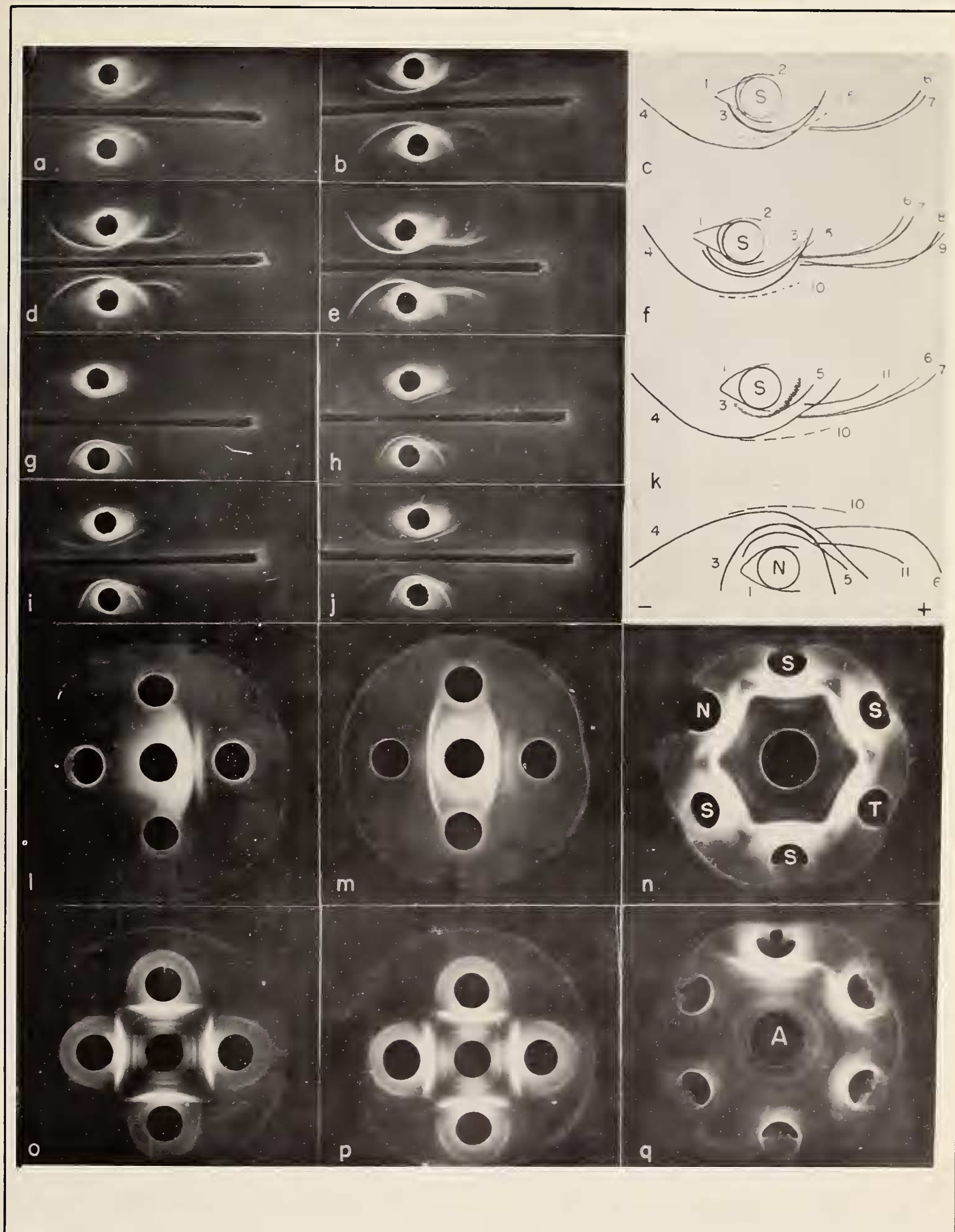
Figure 2.

Immunoelectrophoretic and micro-Ouchterlony double diffusion results with immune ascitic fluid and rabbit antisera.

a-k Results of concentrating immune ascitic fluid. The antigens in both wells in a to f are from *Semisulcospira libertina*. In g to k the upper well contained antigen from *S. libertina* (S) while the lower well contained antigen from *S. niponica* (N). a) "Medium" ascitic fluid from Experiment 8, unconcentrated (41 mg/ml protein). b) Ascitic fluid in "a" concentrated by vacuum dialysis (53 mg/ml). c) Diagrammatic presentation of precipitin systems in "a" and "b"; bands 1, 2 are artifacts found in using the control sera. All bands are strengthened by the concentration, and bands 5 and 7 were made observable. d) "Strong" ascitic fluid from Experiment 8, unconcentrated (52 mg/ml). e) The same (d) concentrated by vacuum dialysis (79 mg/ml). f) The diagrammatic presentation is given for reactions in "d" and "e." Band 4 is notably strengthened by concentration. Bands 3 and 5 are too dense, causing lack of clarity of the systems. Bands 6 and 8 are made evident by the concentration. g) Unconcentrated "medium" immune ascitic fluid from one mouse in Experiment 5. h) Fluid in "g" lyophilized and reconstituted to original volume with distilled water. i) Vacuum dialysis of fluid in "g" to half the original volume. j) Fluid in "g" lyophilized and reconstituted to half the original volume with distilled water. k) diagrammatic presentation of reactions g, h, i, j. Concentration resulted in bands 6, 7 and 11 becoming clearly discerned and making band 10 observable. There were no differences in the results between different methods for concentrating the ascitic fluid. Note that band 3 is clearly delineated in the heterologous reaction while blurred in the homologous reaction.

l-n Results of initial experiments with mouse ascitic fluid containing antibodies in micro-Ouchterlony double diffusion systems. l) Antigens in center well, ascitic fluid (from Experiment 1) full strength in right well, other wells filled with control ascitic fluids from three different mice. m) Center well with antigens (6 mg/ml-protein); right well, ascites (from Experiment 1) half strength; left well, ascites quarter strength; upper and lower wells, rabbit antisera full strength. There were five bands in common and one very pronounced band unique to the ascites systems. n) Center well with ascites (from Experiment 1) half strength. Outer wells with antigens (6 mg/ml-protein). S, *S. libertina* antigens; T. *S. trachea* antigens (*S. trachea* is a synonym of *S. libertina;* see DAVIS, 1968b); N, *S. niponica* antigens. The ascitic fluid is "strong" and yielded seven precipitin systems. No qualitative differences between antigens were noted.

o-q Micro-Ouchterlony double diffusion system with three different rabbit antisera with interfacial ring test titers of 1:1024. In q, there was a serial dilution of antigens (twofold) with a protein concentration of 6 mg/ml in the upper well. Seven precipitin systems resulted. A = antiserum.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tion to using five different rabbit sera, eight pools of ascitic fluid from Experiments 5 through 8 (Tables 11–16) were employed.

D. Concentration of Ascites Fluid—Vacuum dialysis (described handily by CLAUSEN, 1969) was used to concentrate "medium" pools of Experiment 5 using S&S Callodion Bags No. 100 (Schleicher & Schuill, Inc., Keene, N.H., U.S.A.) which retain proteins ⋝ 70,000 mol wt. Dialysis was against physiological saline. The capacity of the bags is 8 ml and the ascites was concentrated to approximately half volume. For comparison with vacuum dialysis, sera were lyophilized and reconstituted with ½ volume of distilled water, reconstitution with original volume (control 1); or used unaltered (control 2). Results of concentrating the ascitic fluid were checked by immunoelectrophoresis.

RESULTS

1. The relative quality of immune ascitic fluid.

It was found that the interfacial ring test did not give an adequate indication of titer relative to the excellent results with this test using rabbit sera. Low titers of 1:32 and 1:64 were obtained although the strength of precipitating systems in gel diffusion indicated a strength comparable to 1:512 or 1:1024 using rabbit antisera. As a result, the quality of the immune ascitic fluid was determined by assessing the number and strength of antigen-antibody precipitating systems in five-well micro-Ouchterlony double diffusion plates. As shown in Figure *1*, a to e are the result of using "strong" ascitic fluid and compare favorably with results using rabbit sera with titers ⋝ 1:512 (compare with Figure *2*, o-p; likewise compare Figure *2*, n with Figure *2*, o-q). Results with some "strong" ascitic fluids (Figure *1*, a-b) indicate quantities of antibody far in excess of that found in any rabbit serum to date; i.e., with titers greatly in excess of 1:1024. "Medium" ascites yielded results as shown in Figure *1*, f-i, while "weak" ascites yielded results comparable to those shown in Figure *1*, j-l.

2. Utility and methodology of concentrating hyperimmune fluid.

There was no observed difference in the immunoelectrophoretic results of concentrating hyperimmune ascitic fluid using vacuum dialysis, or lyophilization and reconstitution to half the original volume with distilled water. When these procedures were done using "medium" ascitic fluid (Figure *2*, g-k), bands 6, 7 and 11 became clearly discernible and band 10 appeared. With these concentrating techniques it was found that the heterologous reaction (involving *Semisulcospira niponica* extract) lacked band 7 (Figure *2*, k).

In another case (Figure *2*, a-c), concentration of "medium" ascitic fluid from Experiment 8 by vacuum dialysis increased the protein concentration from 41 mg per ml to 53 mg per ml with the result that all bands were strengthened and bands 5 and 7 became evident. When "strong" ascitic fluid from the same experiment was similarly concentrated (from 52 mg per ml to 79 mg per ml) bands 6 and 8 were made evident. However, bands 3 and 5 became too dense and thus lost clarity.

Table 3

Injection schedule for eight male mice in a pilot experiment to obtain high titer ascitic fluid. The antigen was from *Semisulcospira libertina*.

	Experimental group (4 mice)			Control group (4 mice)		
Day of injection	Material injected	mg Protein injected/ mouse	ml Fluid injected	Material injected	mg Protein injected/ mouse	ml Fluid injected
1	a + ad	1.1	1.0	s + ad	0	1.0
14	s + ad	0	0.5	s + ad	0	0.5
28	a + ad	1.1	0.6	s + ad	0	0.6

a = lyophilized antigen
ad = adjuvant
s = saline

In subsequent immunoelectrophoretic experiments, ascites from Experiment 5 was concentrated by lyophilization and reconstitution with distilled water to one-half the original volume.

3. Results of initial Experiments 1–5.

(Tables 3–12)

Experiments 1 through 5 represent progressive steps toward establishing procedures now routinely used to produce large volumes of high quality precipitating immune ascitic fluid. As a result of Experiment 1, it was evident that high titer ascitic fluid could be obtained. As shown in Figure *2*, l-n, five to seven strong precipitin systems resulted in the homologous reaction even when the ascitic fluid was cut to half concentration. Compare the strength of reaction in Figure *2*, n with that in Figure *2*, q, where rabbit serum (Table 2, with a titer ⋝ 1:1024 at 60 min.) was tested for precipitating properties (antigen full concentration at top well).

Table 4

Record on the volume of ascitic fluid produced in the pilot experiment outlined in Table 3.

Day tapped	No. mice living	No. mice producing	ml Ascites collected	Average production per producing mouse
		Experimental group		
42	3	1	1.5	1.5
52	3	1	10.0	10.0

Total ml produced—11.5
% of mice producing—33
Accumulative production per producing mouse (average)—11.5

Day tapped	No. mice living	No. mice producing	ml Ascites collected	Average production per producing mouse
		Control group		
42	4	4	26.5	6.6
52	4	0	0	0

Total ml produced—26.5
% of mice producing—100
Accumulative production per producing mouse (average)—6.6

In Experiment 2 (Tables 5, 6) particulate antigens were injected on days 1 and 15. The result was poor production of ascitic fluid with only 7 ml maximum accumulative production per producing mouse (maximum in the experimental group) which ended on the 35th day after the first injection. After three weeks many mice became

Table 5

Experiment 2. Injection schedule for 20 male and 19 female mice of the experimental group and 10 each of males and females of the control group. The freshly prepared antigen was from *Oncomelania hupensis nosophora*.

	Experimental group			Control group		
Day of injection	Material injected	mg Protein injected/ mouse	ml Fluid injected	Material injected	mg Protein injected/ mouse	ml Fluid injected
1	wa + ad	3.8	2.0	s + ad	0	2.0
15	wa + ad	3.8	2.0	s + ad	0	2.0

wa = fresh whole foot extract (uncentrifuged, antigen)
ad = adjuvant
s = saline

Table 6

Record of volume of ascitic fluid produced in Experiment 2.

	Females				Males			
Day tapped	No. mice living	No. mice producing	ml Ascites collected	Average production per producing mouse	No. mice living	No. mice producing	ml Ascites collected	Average production per producing mouse
				Experimental group				
28	18	7	33	4.7	19	5	15	3.0
35	15	9	19	2.1	11	2	1	0.5

Total ml produced—52 (♀) 16 (♂)
% of original 19 mice producing (maximum)—47.4 (♀) 25.0 (of 20 original ♂)
Accumulative production (ml) per producing mouse—6.8 (♀) 3.5 (♂)

Day tapped	No. mice living	No. mice producing	ml Ascites collected	Average production per producing mouse	No. mice living	No. mice producing	ml Ascites collected	Average production per producing mouse
				Control group				
28	10	6	37	6.2	10	5	22	4.4
35	6	4	26	6.5	8	2	7	3.5

Total ml produced—63 (♀) 29 (♂)
% of original 10 mice producing (maximum)—60 (♀) 50 (♂)
Accumulative production (ml) per producing mouse—12.7 (♀) 7.9 (♂)

Table 7

Experiment 3. Injection schedule for 15 males and females of the experimental group and five each of males and females of the control group. The antigen was from *Semisulcospira libertina.*

	Experimental group			Control group		
Day of injection	Material injected	mg Protein injected/ mouse	ml Fluid injected	Material injected	mg Protein injected/ mouse	ml Fluid injected
1	fa + ad	2.5	1.0	s + ad	0	1.0
15	fa + ad	2.5	1.0	s + ad	0	1.0
29	fa + ad	2.5	1.0	s + ad	0	1.0

fa = freshly prepared extract (antigen)
ad = adjuvant
s = saline

swollen as if producing ascites. However, palpation indicated that a hard mass had developed in the abdominal cavity. By day 35, several mice developed gross abdominal abscesses. Necropsy and histological examination indicated proliferating fibrous tissue along with a chronic inflammatory response of the liver, intestine and colon. The gross amount of fibrous tissue was correlated with little to no ascitic fluid production. As a result, work with particulate antigens was dropped.

Experiment 3 was similar to Experiment 1 in terms of schedule of injection. However, fresh antigen was used and antigen was injected on day 15. The total yield of ascitic fluid was very low in the experimental group and significantly higher in the control group. Production of ascites by experimental animals after day 43 was extremely poor. The ascitic fluid produced was "strong" (seven of eight experimental animals).

Experiment 4 was initiated to test Sarcoma 180 relative to producing a large volume of ascitic fluid (Table 9). The injection schedule was somewhat similar to that in Experiment 2. Lyophilized antigen was used. Sixty per-

Table 8

Record of the volume of ascitic fluid produced in Experiment 3.

	Females				Males			
Day tapped	No. mice living	No. mice producing	ml Ascites collected	Average production per producing mouse	No. mice living	No. mice producing	ml Ascites collected	Average production per producing mouse
Experimental group								
28	15	5	3.2	0.6	14	2	3.5	1.8
43	12	3	10.5	3.5	12	3	7.3	2.4
50	11	0	0	0	11	0	0	0
60	8	0	0	0	11	3	8.3	2.7

Total ml produced—13.7 (♀) 19.1 (♂)
% of original 15 mice producing (maximum)—33 (♀) 20.0 (♂)
Accumulative production (ml) per producing mouse—4.1 (♀) 6.9 (♂)

Day tapped	No. mice living	No. mice producing	ml Ascites collected	Average production per producing mouse	No. mice living	No. mice producing	ml Ascites collected	Average production per producing mouse
Control group								
28	5	2	3.3	1.7	5	2	3.8	1.9
43	5	2	13.0	6.5	5	3	14.0	4.7
50	5	1	14.5	14.5	5	4	17.6	4.4
60	4	2	12.0	6.0	4	3	15.0	5.0

Total ml produced—42.8 (♀) 50.4 (♂)
% of original 5 mice producing (maximum)—40 (♀) 80 (♂)
Accumulative production (ml) per producing mouse—28.7 (♀) 16.0(♂)

cent of the mice produced ascites and a comparatively large volume was collected (58 ml and 120.5 ml from experimental females and males respectively) on the 22nd day after the first injection (Table 10). Of 23 mice producing enough ascites to test by micro-Ouchterlony diffusion procedures, only four produced weak bands, the others were precipitin system negative.

As a result of Experiment 4, the schedule of Experiment 1 (Table 3), which resulted in high quality ascitic fluid, was coupled with a Sarcoma 180 injection eight days after the last injection with antigen. As shown in Table 12, the results were highly satisfactory. Greater amounts of ascitic fluid would have been obtained if tapping had been continued beyond 42 days. As seen in Table 17, 63 percent of the ascites producing females and 60 percent of the males of Experiment 5 yielded usable hyperimmune fluid.

4. Females were superior producers.

Experiment 5 was repeated twice (Experiments 6, 7) to determine if, indeed, the inoculation schedule in Experiment 5 resulted in: 1) females producing more ascites than males, 2) production equal to or greater than 25 ml per producing mouse, 3) more females producing high titer ascites than males, 4) more than 40 percent of the ascites producing mice yielding "strong" or high quality immune precipitating fluid (see Table 17).

As seen in Figure 6, the accumulative average per initial mouse (i.e., ml per mouse in terms of number of mice at the beginning of each experiment) for the three experiments clearly shows that females produced twice the volume yielded by males (experimental and control groups). The difference in volume production by males and females is not due to increased mortality rates for males. After day 54, too few mice remained to give a reliable trend per time period.

The difference in volume produced by males and females is attributed to: 1) Fewer males produce ascites than females. The average number of mice producing ascites in each of the three experiments, considering all "tap" periods, was 9.4 for females and 6.6 for males (experimental groups) and 4.7 vs. 3.0 (control groups). 2) Of the producing mice, females actually did produce more. The accumulative production for producing mice summed up for the three experiments was 138.2 ml for females and 93.2 ml for males (experimental groups) and 129.4 ml vs. 79.1 ml (control groups).

As shown in Table 17, columns 6–8, there was little difference in the percent of mice producing strong and medium ascites. Females produced more "strong" ascites than

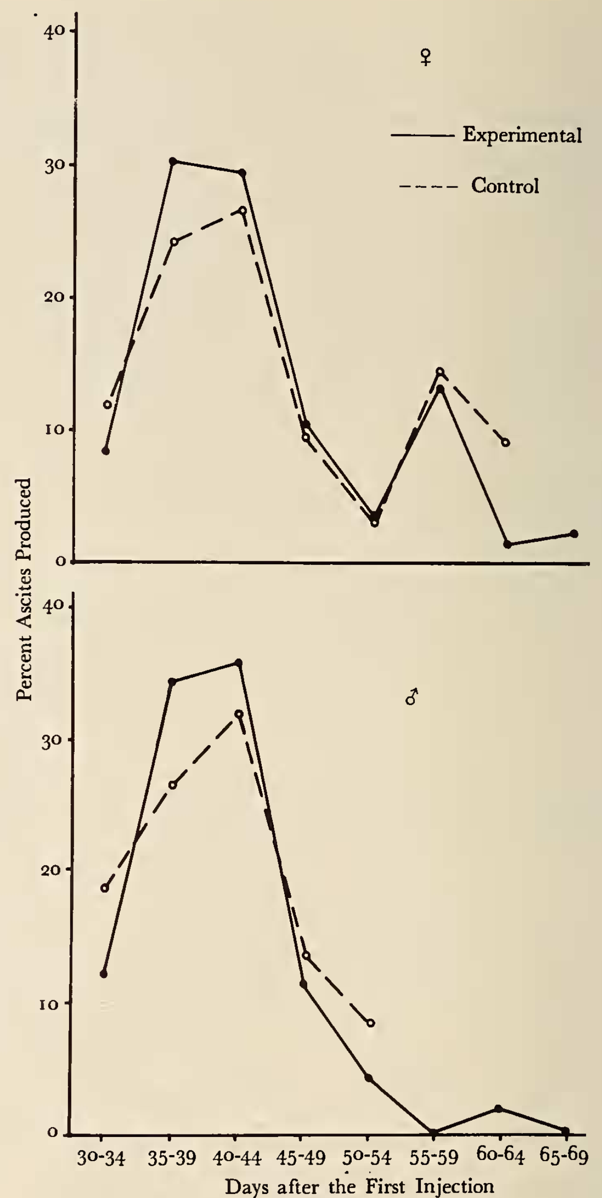


Figure 3

The percentage of ascites produced by mice per unit time following the first injection in Experiments 5 to 7.

males (58% vs. 43%). In only one experiment (No. 5) did mice produce ascites which, throughout several "taps," yielded fluid with no demonstrable precipitating antibodies; these involved only male mice.

5. Time frame for peak production of ascitic fluid, mouse mortality and relative quality of the ascites produced.

As shown in Figure 3, peak production occurred between 35 and 44 days after the first injection when the schedule used in Experiments 5–7 was employed. This pattern was the same for experimental and control groups as well as the sexes. When the mortality of male and female mice was considered (Figure 4), it was seen that 50 percent mortality occurred about 50 days after the first injection. During the period of peak production there was 20 to 30 percent mortality.

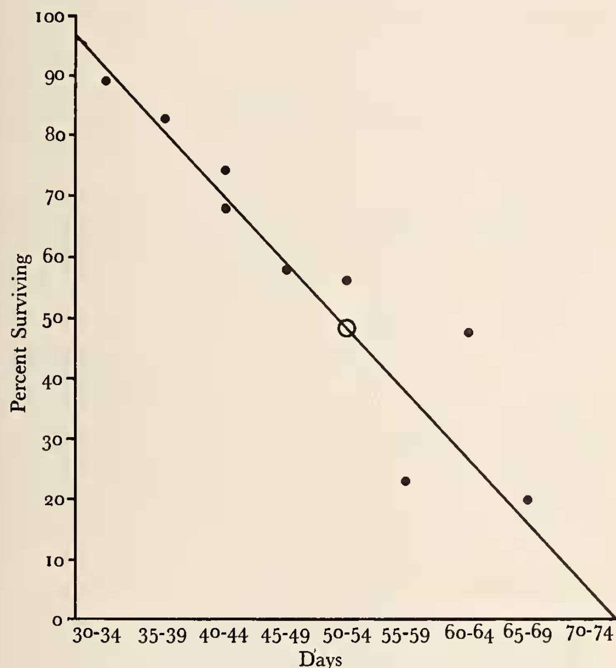


Figure 4

The average percent of experimental male and female mice surviving after the day of the first injection and when Sarcoma 180 was used in Experiments 5 to 7.

About 30 percent of the tap-units produced between days 30 and 34 had no demonstrable precipitating antibodies and an additional seven percent were weak and thus discarded (Figure 5). In the period of peak volume production (days 35 to 44), over 40 percent of the tap-units were "strong" while 21 to 22 percent were weak and had to be discarded.

6. Maximum performance of individual mice.

As shown in Table 18, the greatest volume of ascitic fluid produced by one mouse in one "tap" was 28 ml. The greatest accumulative volume yielded by one mouse was 78 ml. The beneficial effect of using Sarcoma 180 is immediately apparent as seen in the table.

7. The effect of Sarcoma 180 on ascites production; a definitive test.

The results of Experiments 5 to 7 showed that female mice produced more ascitic fluid than males. Experiment 8 was initiated to test the effect of Sarcoma 180 on the volume of ascitic fluid produced. As seen in Table 16, over twice the volume of ascitic fluid is produced in the experimental group when mice receive Sarcoma 180 compared with those not receiving the Sarcoma. In addition, the accumulative production per producing mouse is three times as great. Production using antigen (experimental) is greater than production without antigen (control) in terms of accumulative production per producing mouse. Results with control group C showed that the use of Sarcoma 180 alone, in one injection, resulted in an accumulative production nearly equal to that produced in control group A where mice received saline, adjuvant and Sarco-

Table 9

Experiment 4. Injection schedule for 20 male and female mice of the experimental group and five each of males and females of the control group. The antigen was from *Semisulcospira libertina* and Sarcoma 180 was used.

	Experimental group			Control group		
Day of injection	Material injected	mg Protein injected	ml Fluid injected	Material injected	mg Protein injected	ml Fluid injected
1	a + ad	2.0	0.7	s + ad	0	0.7
16	a + ad + sar	2.0 + sar	0.7	s + ad + sar	0 + sar	0.7

s = lyophilized antigen
ad = adjuvant
sar = Sarcoma 180
s = saline

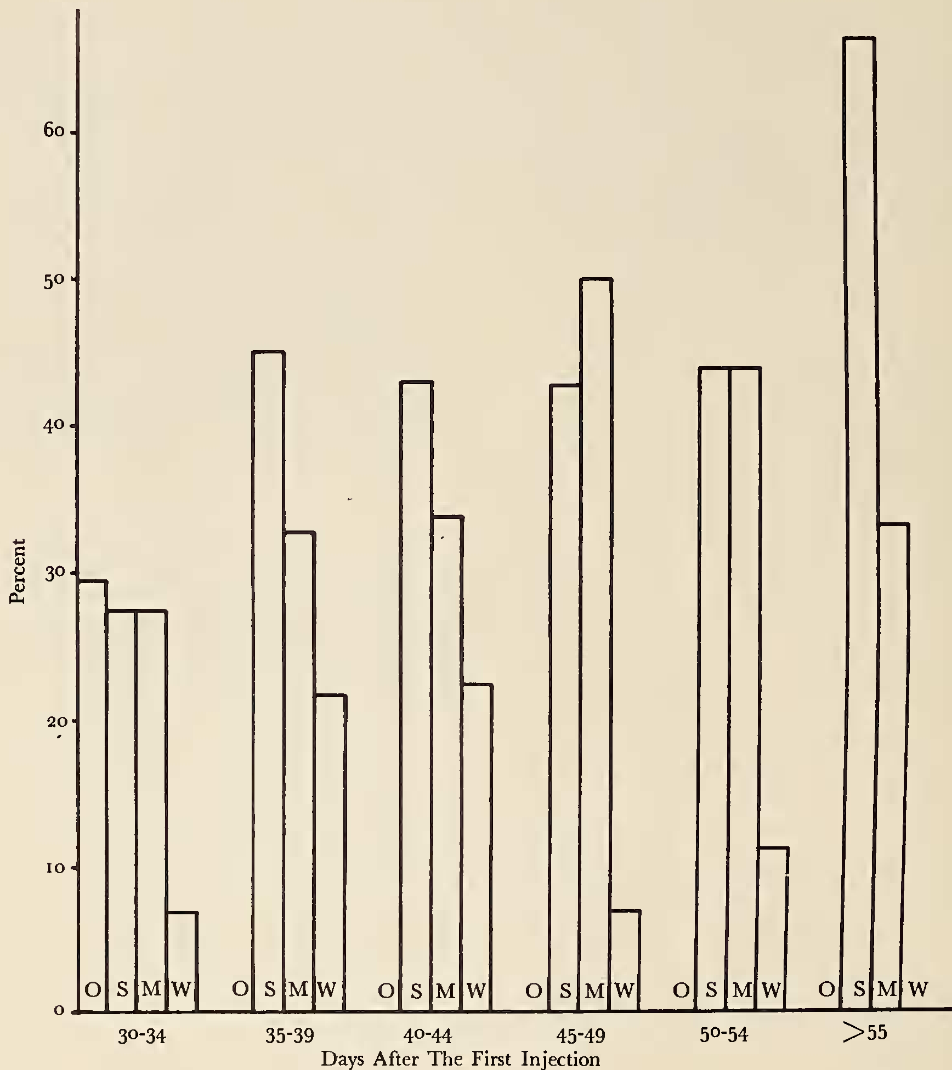


Figure 5

The percent of mice at each time period producing ascitic fluid which lacked demonstrable precipitating antibodies (o), or had strong (s), medium (m) or weak (w) precipitating antibodies. Males and females from Experiments 5 through 7 were used.

ma 180 in a four-shot series. This amount is about half the production when antigen is coupled with Sarcoma 180.

8. Comparing rabbit antisera and mouse ascitic fluid in terms of numbers of discernible precipitating antigen-antibody systems and specificity.

Five rabbit antisera and eight different pools of hyperimmune ascitic fluid are compared in Table 20 in terms of the average number of precipitin systems of the homologous reaction counted from stained slides after the immunoelectrophoretic experiments. It was evident that the same average number of antigen-antibody precipitin systems (arcs or bands) could be obtained using either rabbit or mouse hyperimmune sera (fluids). The greatest average number was 9.3 using rabbit serum and 9.2 using

Table 10

Record of the volume of ascitic fluid produced in Experiment 4, a pilot experiment using Sarcoma 180.

	Females				Males			
Day tapped	No. mice living	No. mice producing	ml Ascites collected	Average production per producing mouse	No. mice living	No. mice producing	ml Ascites collected	Average production per producing mouse
				Experimental group				
22	14	10	58	5.8	14	15	120.5	8.0
% of original mice producing (maximum)—50 (♀) 75 (♂)								
				Control group				
22	3	3	7.5	2.5	4	3	20.5	6.8

% of original mice producing (maximum)—60 (♀) 60(♂)

ascitic fluid. The greatest range (acceptable variation) for both was 7 (8) to 11 (12).

The crudest level of comparison between snail taxa was used to determine the minimum level of specificity of the 13 different hyperimmune sera (fluids). The number of bands in the heterologous reaction was subtracted from the number of bands of the homologous reactions. The remainder was converted to percentage of bands in the homologous reaction "unique" or remaining to the homologous reaction. Taxa were ranked by decreasing affinity to *Semisulcospira libertina* (i.e., decreasing number of pre-

Table 11

Experiments 5 to 7. Injection schedule for 20 male and female mice of the experimental group and 10 of each sex in the control group. The antigen was from *Semisulcospira libertina* and Sarcoma 180 was used.

	Experimental group			Control group		
Day of injection	Material injected	mg Protein injected	ml Fluid injected	Material injected	mg Protein injected	ml Fluid injected
1	a + ad	1.1[1]	0.5	s + ad	0	0.5
14	s + ad	0	0.5	s + ad	0	0.5
28	a + ad	1.1	0.5	s + ad	0	0.5
36[2]	sar	sar	0.5	sar	sar	0.5

a = lyophilized antigen
ad = adjuvant
s = saline
sar = Sarcoma 180
[1]1.0 in Experiments 6 and 7, respectively
[2]Days 32 and 35 in Experiments 6 and 7, respectively

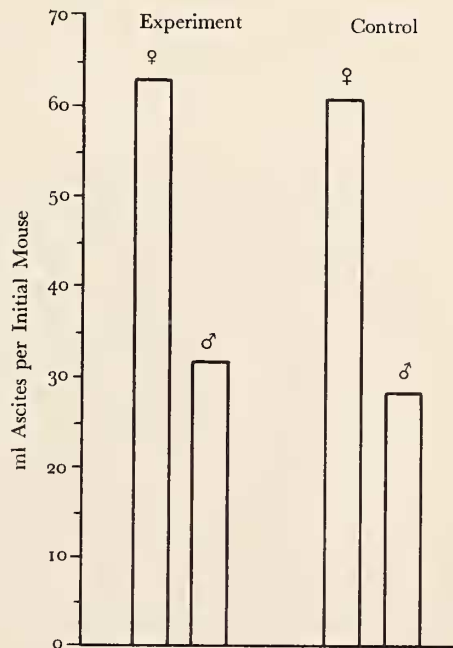


Figure 6

The sum of the average production of ascites produced per initial (= total volume divided by total number of mice initially injected) mouse in Experiments 5 through 7. Divide by three to obtain the average production per mouse per experiment.

Table 12

Record of the volume of ascitic fluid produced in Experiment 5, using Sarcoma 180.

	Females				Males			
Day tapped	No. mice living	No. mice producing	ml Ascites collected	Average production per producing mouse	No. mice living	No. mice producing	ml Ascites collected	Average production per producing mouse
				Experimental group				
32	19	12	54	4.5	20	9	26	2.9
36	19	13	71	5.5	19	10	46	4.6
38	19	11	62	5.6	19	8	42	5.3
40	16	8	48	6.0	17	12	65	5.4
42	14	12	66	5.5	11	9	57	6.3

Total ml produced—301 (♀) 236 (♂)
% of original 20 mice producing (maximum)—65 (♀) 60 (♂)
Accumulative production (ml) per producing mouse—27.1 (♀) 24.5 (♂)

				Control group				
32	10	0	0	0	10	0	0	0
36	10	4	21	5.3	10	2	7	3.5
38	9	3	19	6.3	7	2	13	6.5
40	8	4	24	6.0	6	2	9	4.5
42	7	7	36	5.1	3	3	7	2.3

Total ml produced—100 (♀) 36 (♂)
% of original 10 mice producing (maximum)—70 (♀) 30 (♂)
Accumulative production (ml) per producing mouse—22.7 (♀) 16.8 (♂)

cipitin bands relative to the number in the homologous reaction). As seen in Table 19, there is fairly good agreement between results using rabbit antisera and hyperimmune ascitic fluid in ranking taxa on the average percent of precipitin systems "unique" to the homologous reactions. There was a slight amount of difference in ranking *Batillaria multiformis* (37%), *Melanoides tuberculatus* (37%) and *Thiara scabra* (34%) under results with ascitic fluid relative to results with rabbit sera. As one would suspect, the taxon having closest affinity to *Semisulcospira libertina* is another species of the same genus, *S. niponica*.

An accumulative percent index was constructed by adding up the percentages in each column (i.e., for each serum or fluid) representing those bands "unique" to the homologous reaction in the analysis of each snail taxon. The greatest value represents, in a relative comparative manner, that serum or fluid which was most specific; i.e., yielded the greatest number of antigen-antibody systems "unique" to the homologous reaction. The sera (fluids) are ranked in Table 20 (columns 2-3) in terms of specificity thus determined. As seen in Table 20, the three most specific hyperimmune fluids were from ascitic fluid pools 6s-r, 8m, 7m (accumulative percent indices of 592, 564, 560 respectively, as seen in Table 19). It is thus evident that the greatest specificity of reaction was obtained using hyperimmune mouse ascitic fluid. It is noteworthy that only in cases involving ascitic fluid were differences between *S. libertina* and *S. niponica* detected. Ascitic fluid led rabbit sera on overall average (493 vs. 470, Table 19, columns 7 and 16) in terms of specificity as determined by the accumulative percent index.

Prints of immunoelectrophoretic results are given in Figures 7 and 8. These are representative of 130 sets of reactions involving more than 600 slides. The prints are ar-

Table 13

Record of the volume of ascitic fluid produced in Experiment 6, using Sarcoma 180.

	Females				Males			
Day tapped	No. mice living	No. mice producing	ml Ascites collected	Average production per producing mouse	No. mice living	No. mice producing	ml Ascites collected	Average production per producing mouse
				Experimental group				
34	19	19	232	12.2	14	11	91	8.3
37	16	14	134	9.6	13	9	71	7.9
40	16	11	107	9.7	12	7	44	6.3
44	10	8	127	15.9	8	1	6	6.0
56	7	2	35	17.5	2	0	0	0

Total ml produced—635 (♀) 212 (♂)
% of original 20 mice producing (maximum)—95 (♀) 55 (♂)
Accumulative production (ml) per producing mouse—64.9 (♀) 28.5 (♂)

Day tapped	No. mice living	No. mice producing	ml Ascites collected	Average production per producing mouse	No. mice living	No. mice producing	ml Ascites collected	Average production per producing mouse
				Control group				
34	10	10	79	7.9	7	5	34	6.8
37	10	7	49	7.0	7	5	30	6.0
40	10	9	64	7.1	7	6	32	5.3
44	6	5	35	7.0	6	5	23	4.0
56	3	2	25	12.5	1	0	0	0

Total ml produced—252 (♀) 119 (♂)
% of original 10 mice producing (maximum)—100 (♀) 50 (♂)
Accumulative production (ml) per producing mouse—41.5 (♀) 22.1 (♂)

ranged to represent a series ranging from greatest genetic affinity to *Semisulcospira* to least affinity. The results in Tables 19 and 20 were obtained from an analysis of all of these slides.

DISCUSSION

Wagner & Rasanen (1967) prepared precipitating immune ascites to a variety of vertebrate antigens and obtained excellent results in immunoelectrophoretic experiments. They stated that 10 mice were roughly equal to one rabbit in terms of producing hyperimmune serum. They found that precipitate bands obtained using mouse ascites corresponded favorably with those produced with corresponding immune rabbit sera. The results of this paper, using molluscan antigens, agree fairly well with these authors.

In this laboratory we have invested a minimum of 24 mg protein to immunize one rabbit. The process takes 39 days and the maximum yield was 100 to 125 ml serum. The same production can be obtained using eight to nine female mice and investing 16 to 18 mg protein. This is based on the fact that the average production throughout an experiment, in terms of the initial number of female mice injected, was 19.3 ml (and thus mortality has been accounted for) and over 75 percent of the fluid produced would be strong or medium quality and thus useful. As Wagner and Rasanen pointed out, "for total failure in immunization, up to ten [8–9] individual mice, in lieu of one rabbit, have to be lost . . ."

The use of whole rabbit blood to help clot the freshly drawn ascitic fluid makes up for the volume of ascites lost due to clotting and, in fact, increases the final volume of fluid. The rabbit serum does not affect the results as determined by the number and density of precipitate arcs

Table 14

Record of the volume of ascitic fluid produced in Experiment 7, using Sarcoma 180.

	Females				Males			
Day tapped	No. mice living	No. mice producing	ml Ascites collected	Average production per producing mouse	No. mice living	No. mice producing	ml Ascites collected	Average production per producing mouse
				Experimental group				
35	17	9	103	11.4	19	2	16	8.0
38	14	4	28	7.0	18	6	37	6.2
43	13	9	91	10.1	18	7	61	8.7
49	12	8	61	7.6	16	6	62	10.3
52	9	4	30	7.5	14	4	16	4.0
60	6	3	11	3.6	13	2	4	2.0

Total ml produced—324 (♀) 196 (♂)
% of original mice producing (maximum)—45 (♀) 35 (♂)
Accumulative production (ml) per producing mouse—47.2 (♀) 39.2 (♂)

Day tapped	No. mice living	No. mice producing	ml Ascites collected	Average production per producing mouse	No. mice living	No. mice producing	ml Ascites collected	Average production per producing mouse
				Control group				
35	10	4	50	12.5	10	1	8	8.0
38	10	4	37	9.3	10	1	5	5.0
43	10	5	70	14.0	9	6	55	9.2
49	9	5	40	8.0	8	5	55	11.0
52	8	4	22	5.5	4	1	7	7.0
60	7	2	32	16.0	4	0	0	0

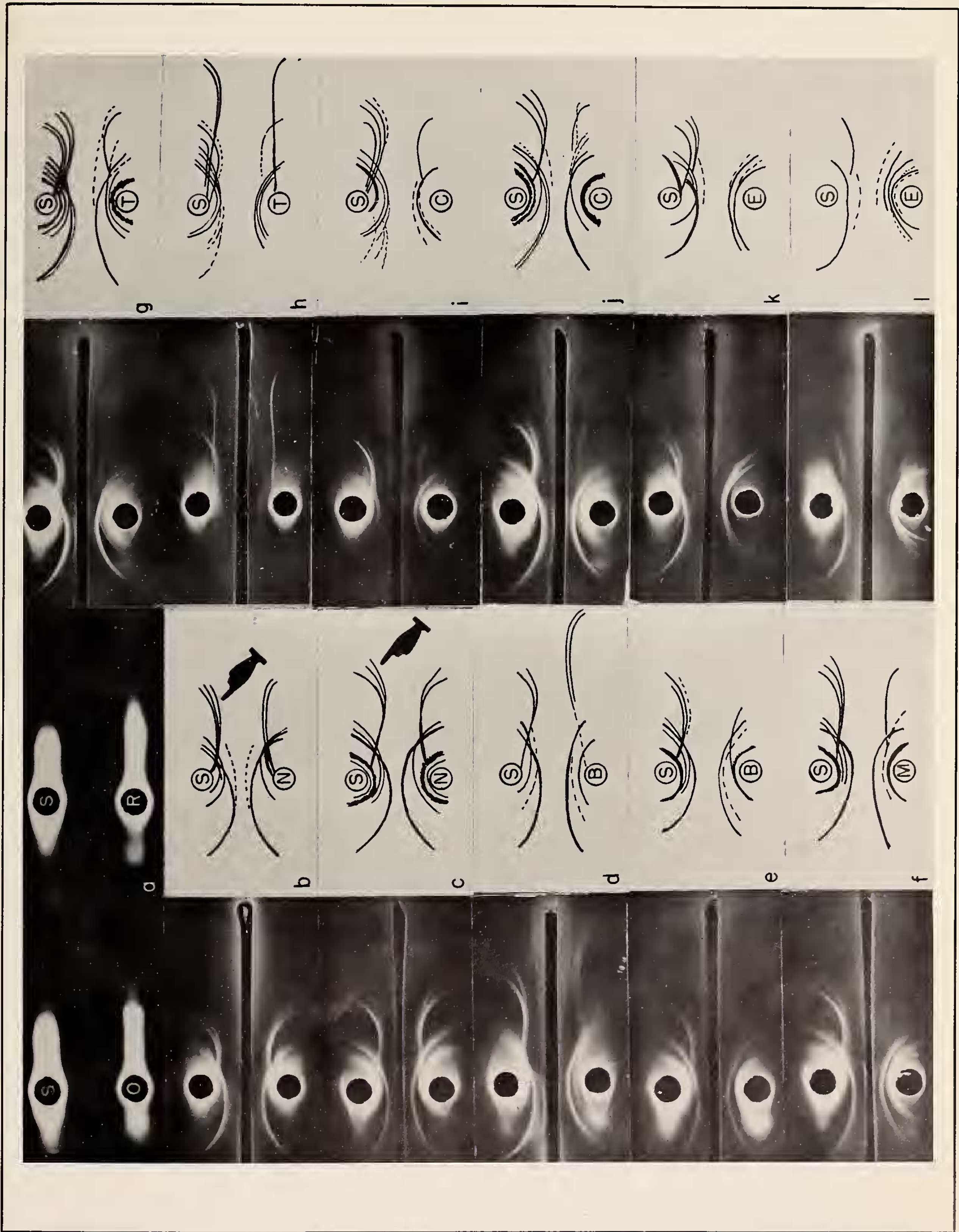
Total ml produced—251 (♀) 130 (♂)
% of original 10 mice producing (maximum)—50 (♀) 60 (♂)
Accumulative production (ml) per producing mouse—65.3 (♀) 40.2 (♂)

Plate Explanation

Figures *7, 8.*

Representative prints of the immunoelectrophoretic results chosen from 130 sets of reactions involving 11 taxa of snails and 13 different sera (or ascites). The prints are arranged to represent a series ranging from greatest genetic affinity to *Semisulcospira* (Figure *1*, b-c) to least affinity (Figure *2*, j-l). The drawn patterns are presented because photographic reproduction of the precipitin systems is often not adequate to make faint precipitin systems visible in print. From Figure *2*, d one can see two bands at the well of the homologous reaction(s) when control ascitic fluid was used. These two bands are artifacts and were not considered in the analysis of the slides. Depending on the experiment there were zero to three such bands. Results in Figure 7a show the migration pattern of the protein in the electric currents at 60 minutes. The greatest bulk of the protein traveled eight to 12 mm from the anodal edge of the well while some protein traveled 18 to 20 mm from the edge of the well (anodally). Hyperimmune fluid was used as follows: Figure 7b, 6s-nr; *7*c, 6s-r; *7*d, 6s-nr; *7*e, 6s-r; *7*f, 6s-r; *7*g, 6s-r; 7h, D; 7i, B; 7j, 6s-r; 7k, 6s-r; 7l, 5c; Figure *8*a, 7m; *8*b, 6s-r; *8*c, C; *8*d, 6-cont; *8*e, 7s; *8*f, 6s-r; *8*g, 6s-r; *8*h, 8m; *8*i, C; *8*j, A; *8*k, 6m; *8*l, 6s-r.

The pointers in Figure 7b and 7c indicate the one precipitin system unique to the homologous reaction, thus indicating a difference between *Semisulcospira libertina* and *S. niponica*. B, *Batillaria multiformis*; C, *Stenomelania crenulata*; E, *Brotia costula episcopalis*; H, *Clypeomorus humilis*; I, *Sinotaia histrica*; M, *Melanoides tuberculatus*; N, *Semisulcospira niponica*; O, *Oncomelania hupensis nosophora*; R, *Clithon retropictus*; S, *Semisulcospira libertina*; T, *Thiara scabra*.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